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Sir:

In response to item #4 of the Notice of Allowability, enclosed herewith is a certified copy of Australian priority application no. PQ8418.

Respectfully submitted,

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I, JANENE PEISKER, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PQ 8418 for a patent by COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION, LUDWIG INSTITUTE FOR CANCER RESEARCH and BIOMOLECULAR RESEARCH INSTITUTE LIMITED as filed on 28 June 2000.

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AUSTRALIA

Patents Act 1990

Commonwealth Scientific and Industrial Research Organisation

Biomolecular Research Institute Limited

Ludwig Institute for Cancer Research

PROVISIONAL SPECIFICATION

Invention Title:

Truncated EGF receptor

The invention is described in the following statement:

Truncated EGF receptor

FIELD OF THE INVENTION

The present invention relates to truncated EGF receptor molecules and to pharmaceutical compositions comprising these molecules. The present invention also relates to methods of screening for EGF receptor ligands and methods of treatment which involve the use of these molecules.

BACKGROUND OF THE INVENTION

The epidermal growth factor receptor (EGFR) family consists of four distinct tyrosine kinase receptors, EGFR/HER/ErbB1, HER2/Neu/ErbB2, HER3/ErbB3 and HER4/ErbB4 (Burgess and Thumwood, 1994). These receptors are widely expressed in epithelial, mesenchymal and neuronal tissues and play fundamental roles during development and differentiation (Burgess and Thumwood, 1994). They are activated by a family of at least twelve ligands that induce either homo- or hetero-dimerisation of the EGFR homologues (Sundaresan et al., 1998). ErbB2 is unable to bind ligand on its own but is a potent co-receptor for all ligands when co-expressed with other members of the EGFR/HER/ErbB family (Klapper et al., 1999).

The EGFR is a large (1,186 residues), monomeric glycoprotein with a single transmembrane region and a cytoplasmic tyrosine kinase domain flanked by noncatalytic regulatory regions. Sequence analyses have shown that the ectodomain (residues 1-621) contains four sub-domains, here termed L1, CR1, L2 and CR2, where L and CR are acronyms for large and Cys-rich respectively (Bajaj et al., 1987; Ward et al., 1995). The L1 and L2 domains have also been referred to as domains I and III, respectively (Lax et al., 1988). The CR domains have been previously referred to as domains II and IV (Lax et al., 1988), or as S1.1 - S1.3 and S2.1 - S2.3 where S is an abreviation for small (Bajaj et al., 1987).

Many cancer cells express constitutively active EGFR (Sandgreen et al., 1990) or other EGFR family members (Hines, 1993). Elevated levels of activated EGFR occur in bladder, breast, lung and brain tumours (Harris et al., 1989). Antibodies to EGFR can inhibit ligand activation of EGFR and the growth of many epithelial cell lines. Patients receiving repeated doses of a humanised chimeric anti-EGFR monoclonal antibody (Mab) showed signs of disease stabilization. The large doses required and the cost of production of

humanised Mab is likely to limit the application of this type of therapy. These findings indicate that the development of EGF receptor antagonists will be attractive anticancer agents.

SUMMARY OF THE INVENTION

The present inventors have now made the surprising finding that the deletion of residues in the CR2 domain of an EGFR ectodomain gives rise to a truncated ectodomain with enhanced affinity for EGF and/or TGF-α. This finding goes against recent results reported by Saxon and Lee (1999) showing that deletions or mutations in the CR2 region reduce EGFR binding affinity for EGF.

As will be appreciated by those skilled in the art, the truncated EGFR ectodomains of the present invention may provide increased sensitivity in assays which screen for ligands of the EGF receptor. Furthermore, the truncated EGFR ectodomains of the present invention may have therapeutic potential given their high affinity for ligand and their ability to competitively inhibit EGF-induced proliferation responses *in vitro*.

Accordingly, in a first aspect the present invention provides a truncated EGFR ectodomain, the truncated EGFR ectodomain lacking a substantial portion of the CR2 domain such that the truncated EGFR ectodomain has an increased binding affinity for at least one EGFR ligand when compared to the full length EGFR ectodomain.

In a preferred embodiment of the first aspect the truncated EGFR ectodomain has an increased binding affinity for EGF and/or TGF-α.

In a further preferred embodiment of the first aspect the truncated EGFR ectodomain lacks at least the third to seventh modules of the CR2 domain. In a further preferred embodiment, the truncated EGFR ectodomain lacks at least the second to seventh modules of the CR2 domain. The truncated EGFR ectodomain may further lack a portion of the first module of the CR2 domain.

In a further preferred embodiment, the truncated EGFR ectodomain lacks residues 514-621. In a further preferred embodiment, the truncated EGFR ectodomain lacks residues 502-621.

Further deletions or mutations may be made to the L1, CR1 and/or L2 sub-domains of the truncated EGFR ectodomain of the present invention, provided that these further deletions or mutations do not substantially affect

the binding affinity of the truncated EGFR ectodomain. Preferably, however, the truncated EGFR ectodomain of the present invention comprises the L1, CR1 and L2 subdomains.

In a further preferred embodiment, the truncated EGFR ectodomain comprises residues 1-492 of the EGFR ectodomain. More preferably, the truncated EGFR ectodomain comprises residues 1-501 or residues 1-513 of the EGFR ectodomain.

In a further preferred embodiment, the truncated EGFR ectodomain has an affinity for EGF such that the K_d is less than 30 nM, more preferably less than 25 nM.

In a further preferred embodiment, the truncated EGFR ectodomain has an affinity for TGF-α such that the K_d is less than 45 nM, more preferably less than 40 nM.

In a second aspect, the present invention provides a pharmaceutical composition comprising a truncated EGFR ectodomain according to the first aspect and a pharmaceutically acceptable carrier or diluent.

In a third aspect, the present invention provides a method of screening a putative compound for the ability to modulate the activity of the EGF receptor, the method comprising exposing the putative compound to a truncated EGFR ectodomain according to the first aspect and monitoring the activity of the truncated EGFR ectodomain.

In the context of the third aspect, a suitable assay procedure may involve a competition binding assay in a microplate format, where the putative compound is tested for its ability to inhibit the binding of labelled ligand such as IGF-1 or IGF-2 to the truncated EGF receptor ectodomain. The label may be a radiolabelled tag such as 125I or a fluorescent tag such as fluorescein isothiocyanate or a lanthanide ion such as europium.

In a fourth aspect the present invention provides a method of treating or preventing a disease associated with signalling by a molecule of the EGF receptor family in a subject, the method comprising administering to the subject a truncated EGFR ectodomain according to the first aspect.

In a preferred embodiment of the fourth aspect, the disease associated with signalling by a molecule of the EGF receptor family is selected from psoriasis and tumour states comprising but not restricted to cancer of the breast, brain, ovary, cervix, pancreas, lung, head and neck, and melanoma, rhabdomyosarcoma, mesothelioma and glioblastoma.

The method of the fourth aspect may be used alone or in combination with other therapeutic measures. For example, the method of the fourth aspect may be used in combination with cytotoxic modalities, such as radiotherapy or chemotherapy, in the treatment of tumour states.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Stereoview of residues 301-501 of a model of EGF receptor domains L2 and CR2 (Jorissen et al., 2000) shown in trace representation. The Ca atoms of selected atoms are shown as spheres and numbered. The trace of residues 351-364, which form an epitope for three ligand-competitive antibodies (Wu et al., 1989) is shown as a dashed line.

Figure 2: Downstream oligonucleotides used in PCR for construction of the human EGFR truncated to residues 476, 501 or 513. The primer sequences are complementary to the last six codons of the truncated receptor and the attached C-terminal tail which codes for an enterokinase (EK) cleavage site and a c-myc epitope tag to aid purification (McKern et al 1997). Nucleotides 1744 to 1760 or 1780 to 1797 of hEGFR cDNA and the corresponding receptor sequences are in bold. The inner primers overlap the outer primer by 16 nucleotides as shown.

Figure 3: Gel filtration chromatography of purified human sEGFR501 on TSK G2000SW. sEGFR501 purified by affinity chromatography on an anti c-myc Mab 9E10 column was analysed by micropreparative SEC HPLC using a TSK G2000SW installed in a HP1100 HPLC. The column temperature was 25°C and the flow rate was 250µl/min. Fractions were recovered manually at 1min intervals. The arrows indicate the elution positions of protein standards. Insert: SDS-PAGE analysis (8-25% non-reducing gel) of aliquots (5µl) from the fractions indicated. Detection was by silver staining.

Figure 4: Analysis of EGF/sEGFR501 interactions using the analytical ultracentrifuge. (A) Sedimentation equilibrium analysis of EGF, sEGFR501 and a mixture of EGF and sEGFR501. The equilibrium distributions were obtained after centrifugation at 12,000 rpm at 20°C for 16 h. (□) 20 μM EGF; (O) 10 μM sEGFR501, (Δ) 20 μM EGF + 10 μM sEGFR501. The lines of best fit drawn though the data for EGF and sEGFR501 are for single species and for molecular weight values of 6,000 and 65,600 respectively. The line drawn through the data for the EGF/sEGFR501 mixture is the line of best-fit calculated assuming two species with the molecular weight of the first species fixed at 6,000 and a fitted value of 106,400 for the molecular weight of the second species. (B) Meniscus depletion sedimentation analysis of the stoichiometry of EGF binding to sEGFR501. Solutions containing 5 μM sEGFR501 and different molar ratios of EGF:EGFR were spun for 16 h at 20,000 rpm and 20°C in the XLA analytical ultracentrifuge. Under these conditions sEGFR501 and its complexes with EGF are depleted from the meniscus leaving unbound EGF in the supernatant. Optical density measurements at 280 nm enable the amount of unbound EGF near the meniscus to be estimated. (C) Data obtained for the weight-average molecular weight of the "second" species calculated for mixtures of sEGFR501 (5 μM) and EGF at the concentrations indicated.

Figure 5: Covalent cross-linking of sEGFR501 dimers after incubation with mEGF. sEGFR501 (5 μM) was incubated with (+) or without (-) mEGF (20 μM) in 20 mM HEPES (pH7.4) containing 150 mM NaCl for 1 h at room temperature followed by the addition of bis(sulfosuccinimidyl)suberate (BS3, Pierce, Rockford, IL, USA) to a final concentration of 0.5 mM and incubation for a further 30 min. The reaction was terminated and the degree of dimer formation was monitored by SDS-PAGE and immunoblotting with anti-EGFR Mab528 (Gill et al., 1984) (0.5 μg/ml) and horseradish-peroxidase labelled goat anti-mouse IgG (Bio-Rad) with detection by ECL (Amersham Pharmacia Biotech).

Figure 6: BIAcore analysis of the interactions between sEGFR501 and sEGFR621 with immobilised hEGF or hTGF-α. (A): sEGFR501 (140, 120, 100, 80, 60 and 40 nM) was passed over immobilised hEGF (160 RU immobilised). Samples (30 μl) were injected at a flow rate of 10 μl/min. (B): sEGFR501 was

passed over immobilised hTGF-α 132 RU immobilised). Experimental details were as in Figure 6A. (C): sEGFR621 (1000, 900, 800, 700, 600 and 500 nM) was passed over immobilised hEGF . (D): EGFR501 (concentrations as for Figure 6C) was passed over immobilised hTGF-α. The operating temperature was 25°C. At the end of the injection phase, dissociation was monitored with buffer alone flowing over the sensor surface. The surface was regenerated between samples using 10 mM HCl. The signal obtained when the sample was passed over a parallel blank channel has been subtracted electronically to give the specific response.

Figure 7: Scatchard analysis of equilibrium binding data. The dissociation constant (KD = 1/KA) was calculated from the equilibrium binding response obtained in Figure 6 by plotting the data in Scatchard format (Req/nC versus Req; see Experimental Procedures) The slope of the linear fit to the data gives KA. (A): sEGFR501 versus hEGF (B): sEGFR501 versus hTGF-α. (C): sEGFR621 versus hEGF (D): sEGFR621 versus hTGF-α.

Figure 8: Inhibition of EGF-stimulated cell mitogenesis by sEGFR501. (A): The stimulation of 3H-thymidine incorporation by BaF/3ERX cells using serial dilutions of mEGF. The data was fitted by a sigmoidal function (-) to determine the EC50. (B): Inhibition of the mitogenic response of BaF/3ERX cells stimulated with mEGF (207 pM) by : sEGFR501 (■ - ■), sEGFR621 (● - ●) or Mab528 (▲ - ▲). Each point was assayed in triplicate. Error bars are shown.

Figure 9: BIAcore analysis of sEGFR501 mutants. Tissue culture media from transiently transfected cells (25 µl) expressing wild type sEGFR501 (501), the Gly441Lys mutant (441) or the Glu472Lys mutant (472) of sEGFR501 were injected over parallel hEGF (panel A) and hTGF-α surfaces (panel B) at a flow rate of 10 µl/min. The blank refractive index signal obtained with non-transfected control media has been subtracted electronically using BIAevaluation 3.0 to give the specific response.

DETAILED DESCRIPTION OF THE INVENTION

When used herein, the phrase "full length EGFR ectodomain" refers to the ectodomain consisting of residues 1-621 of the EGF receptor. The amino acid sequence of the full length ectodomain is described in Ullrich et al., 1984 Nature 309, 418-425. The full length ectodomain contains four sub-domains, referred to as L1, CR1, L2 and CR2, where L and CR are acronyms for large and cys-rich respectively.

The CR2 sub-domain consists of the following seven modules joined by linkers of 2 or 3 amino acid residue and bounded by the cysteine residues as follows:

First module:	cys residues 482-499
Second module:	cys residues 502-511
Third module:	cys residues 515-531
Fourth module:	cys residues 534-555
Fifth module:	cys residues 558-567
Sixth module:	cys residues 571-593
Seventh module:	cys residues 596-612.

The results presented herein show that deletions in the CR2 region of the EGFR ectodomain unexpectedly increase binding affinity of the ectodomain for EGF and/or TGF-α. In light of this information, a person skilled in the art would be able to readily generate a number of candidate truncated ectodomains and screen these candidates for increased ligand affinity and for therapeutic potential.

For example, truncated ectodomains may be prepared by recombinant DNA technology as described herein or as described in Saxon and Lee (1999). Alternatively, truncated ectodomains may be prepared by subjecting the full length frgament to limited proteolysis as described in Kohda et al (1993).

Binding affinity and inhibitor potency may be measured for candidate truncated ectodomains using biosensor technology.

Truncated EGFR ectodomains may be tested for their ability to modulate receptor activity using a cell-based assay incorporating a stably transfected, EGF-responsive reporter gene (Souriau, C., Fort, P., Roux, P., Hartley, O., Lefranc, M-P., Weill, M., 1997, Nucleic Acids Res. 25:1585-1590). The assay addresses the ability of EGF to activate the reporter gene in the presence of novel ligands. It offers a rapid (results within 6-8 hours of

hormone exposure), high-throughput (assay can be conducted in a 96-well format for automated counting) analysis using an extremely sensitive detection system (chemiluminescence). Once candidate compounds have been identified, their ability to antagonise signal transduction via the EGF-R can be assessed using a number of routine in vitro cellular assays such as inhibition of EGF-mediated cell proliferation. Ultimately, the efficiency of truncated EGFR ectodomains as tumour therapeutics may be tested *in vitro* in animals beating tumour isografts and xenografts as described (Rockwell, P., O'Connor, W.J., King, K., Goldstein, N.I., Zhang, L.M., Stein, C.A., 1997, Proc Natl Acad Sci U S A 94:6523-6528; Prewett, M., Rothman, M., Waksal, H., Feldman, M., Bander, N.H., Hicklin, D.J., 1998 Clin Cancer Res 4:2957-2966).

EXPERIMENTAL DETAILS

MATERIALS AND METHODS

Construction of plasmids used for the expression of truncated forms of hEGFR ectodomain - The plasmid, pEGFR, used in the construction of truncated hEGFR cDNAs comprised nucleotides 167-3970 of hEGFR (Ullrich et al., 1984) in the multiple cloning site of plasmid pUC18. Coding was in the opposite sense to the LacZ α peptide, and the insertion was downstream of the *Xba*I site of pUC18, which was used later in excision of the truncated constructs for insertion into the mammalian expression vector pEE14 (Bebbington and Hentschel, 1987).

Construction of pEGFR476 - An initial plasmid containing nucleotides 167–3150 of hEGFR was constructed by ligation of a *Xba*I/*Nsi*I fragment from pEGFR and *Xba*I/*Pst*I-cut pBluescript KS+. From this plasmid, a 4 kbp fragment *Bbs*I/*Bgl*II fragment (containing all of pBluescript KS+ and nucleotides 167-1150 and 2951-3150 of EGFR) and a 528 bp *Bbs*I/*Pvu*II fragment (nucleotides 1151-1679) were ligated with a 70 bp PCR-derived *Pvu*II and *Bgl*II fragment, encoding amino acids 474-476 of hEGFR, an enterokinase cleavage site and a c-myc epitope tag to facilitate purification (Figure 2). The 70 bp PCR cassette was produced using a similar previous construct (McKern et al., 1997) as template. A plasmid for mammalian cell

transfection, pEGFR476, was constructed from this plasmid by ligation of a 1.6 kbp *XbaI/Eco*RV fragment with *XbaI/Sma*I-cut pEE14.

Construction of pEGFR501and pEGFR513- In each construction PCR was used with three oligonucleotides to produce a fragment of hEGFR cDNA (nucleotides 1121 to 1760 or 1121 to 1797 respectively), followed by sequence which encoded an enterokinase cleavage site, a c-myc epitope tag and a termination codon. The upstream primer in PCR corresponded to an arbitrary choice of nucleotides 1121-1140 of hEGFR cDNA, while two overlapping downstream primers were used to construct additional sequence adjacent to nucleotide 1760 or 1797 respectively (Figure 2). The PCR products were cloned using the pCR-Script vector (Stratagene). In each case this allowed an *Apa*I fragment harbouring the newly constructed sequence beginning at nucleotide 1738 of hEGFR, to be excised for subsequent insertion into the large *Apa*I fragment of pEGFR (which included the entire pUC18 sequence with hEGFR cDNA to nucleotide 1737), in order to prepare a plasmid encoding a truncated hEGFR with *Xba*I restriction sites adjacent to the coding sequence. From these pUC18-based plasmids the fragments harbouring the truncated hEGFR cDNAs were excised by *XbaI* digestion, and inserted into plasmid pEE14 at the *Xba*I site to prepare plasmids pEGFR501 and pEGFR513 respectively for mammalian cell transfection.

Mutagenesis – The 1.7 kbp fragment harbouring the truncated hEGFR cDNA of pEGFR501 was introduced into M13mp18 (Norrander et al., 1983) for mutagenesis. Oligonucleotide-directed in vitro mutagenesis, using the USB-T7 Gen™ *in vitro* mutagenesis kit, was employed to produce three single site mutants of the truncated human sEGFR501 ectodomain, with residues Glu367, Gly441 and Glu472 respectively mutated to Lys to match the corresponding residues in chicken EGFR (Lax et al., 1988). Clones incorporating the mutations were identified by colony hybridisation (Carter, 1987) using ^{32}P-labelled mutagenic oligonucleotide as a probe, and the mutations were confirmed by DNA sequence analysis (Sanger et al., 1977). Vehicles for mammalian cell expression were generated for each mutant by excising the 1.7 kbp fragment harbouring the mutated sEGFR501 cDNA from M13 RF-DNA by *Xba*I digestion and inserting it into plasmid pEE14 (Bebbington and Hentschel, 1987) at the *Xba*I site.

Cell Culture, DNA Transfection and Protein Analysis - For transient transfection assays; human 293T fibroblasts maintained in DMEM plus 10% fetal calf serum (FCS) were transfected with plasmid DNA using FuGENE (Roche Molecular Biochemicals, Sydney, NSW) according to the manufacturers instructions. Supernatants were harvested 48 h after transfection, and cell lysates were prepared in NP-40 lysis buffer (Harlow and Lane, 1988). To characterise secreted EGFR mutants, aliquots of supernatant and lysate were immunoprecipitated with a monoclonal antibody (9E10) to the c-myc tag, or with Mab 225 (HB-8508, American Type Culture Collection), a monoclonal antibody for the extracellular domain of the hEGFR (Gill et al., 1984). Immune complexes were collected on protein A-Sepharose beads (Zymed Laboratories, Bioscientific Pty. Ltd., Gymea, NSW), fractionated by SDS polyacrylamide gel electrophoresis (10% gel) and transferred to nitrocellulose membranes (Harlow and Lane, 1988). Truncated hEGFR ectodomains and mutants were identified by probing membranes with horseradish peroxidase (HRP)-conjugated Mab9E10 (Roche), followed by chemilumiscent detection with Pierce Super Signal substrate.

Stable cell lines expressing sEGFR501 were established in the Lec 8 mutant cell line from CHOK (Stanley 1989) using glutamine synthetase as a selectable marker (McKern et al., 1997). Supernatants from methionine sulfoximine (MSX)-resistant cell colonies were screened for secreted receptor by biosensor analysis (see below) or by dot-blotting onto nitrocellulose and probing with HRP-Mab9E10. A single cell line (501-72) was selected for cloning by limiting dilution.

Production and Purification of Truncated EGFR Ectodomains - Lec8 cells expressing sEGFR501 protein were cultured in a Celligen Plus bioreactor (New Brunswick Scientific, New Jersey, USA) using 70 g Fibra-Cell Disks carriers with 1.7L working volume. Continuous perfusion culture using glutamine-free DMEM/F12 medium supplemented with non-essential amino acids, nucleosides and 10% FCS was maintained for 6 weeks. Selection pressure was maintained with 25 μM MSX for the duration of the fermentation. Perfusion rate was adjusted as required to ensure a residual glucose level of 1.0-1.5 g/L, with a corresponding lactate concentration of 2.0-2.3 g/L.

Conditioned medium containing sEGFR501 protein was adjusted to pH 8.0 with Tris-HCl (Sigma), sodium azide was added to 0.02% (w/v), and particulates were removed by centrifugation prior to the recovery of c-myc-tagged protein by affinity purification at 4°C using a column of monoclonal antibody 9E10, covalently-bound to agarose and peptide elution (McKern et al., 1997). Eluted protein was purified by size exclusion chromatography in TBSA on Superdex 200 (HR10/30 Amersham Pharmacia Biotech) at room temperature with a flow rate of 0.8 ml/min. Protein was detected by absorbance at 280nm.

BIAcore Binding Assays – Protein-protein interactions were monitored in real time using an instrumental optical biosensor (BIAcore 2000 or 3000, BIAcore, Uppsala, Sweden). Recombinant hEGF or hTGF-á (Gropep, Adelaide, Australia) were purified immediately prior to immobilisation by micropreparative RP-HPLC using a SMART system (Amersham Pharmacia Biotech) as described previously (Nice et al., 1994). The proteins were immobilised onto the biosensor surface using amine coupling chemistry (N-hydroxysuccinimide and N-ethyl-N'-dimethylaminopropyl-carbodiimide) at a flow rate of 4 μl/min. Automated targetting of immobilisation levels was achieved using the BIAcore 3.1 control software (Catimel et al, 1999).

Prior to kinetic analysis, sEGFR501 samples were characterised by micropreparative size exclusion chromatography (Superose 12 3.2/30, AmershamPharmacia Biotech) to ensure size homogeneity (Nice et al. 1994) and pooled fractions were diluted in BIAcore buffer (HBS: 10 mM Hepes pH 7.4 containing 3.4 mM EDTA, 0.15 mM NaCl and 0.005% (v/v) Tween 20) to the appropriate concentration. Typically, samples (30 μl) were injected over the sensor surface at a flow rate of 5 or 10 μl/min. Following completion of the injection phase, dissociation was monitored in BIAcore buffer for 150 sec at the same flow rate. The sensor surface and sample blocks were maintained at 25°C. Bound receptor was eluted, and the surface regenerated between injections, using 40 μl of 10 mM HCl. This treatment did not denature hEGF or hTGF-α immobilised onto the sensor surface, as shown by equivalent signals on re-injection of receptor.

Kinetic rate constants (k_a, k_d) were determined using the BIAevaluation 3.02 software (BIAcore, http//www.biacore.com/ products/eval3.html) as described previously (Catimel et al., 1997), or by global analysis using

CLAMP (Morton and Myszka, 1998). Equilibrium binding constants (KA, KD) were determined by direct non-linear least squares analysis of the binding data using an equation defining steady state equilibrium (KA*Conc*Rmax/(KA*Conc*n-1); BIAevaluation 3.1). The data was also plotted in Scatchard format (Req/nC versus Req, where Req is the biosensor response at equilibrium, n is the valency and C is the concentration) (Hammacher et al., 1996).

Analytical Ultracentrifugation – Experiments were performed using a Beckman XL-A analytical ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA) equipped with absorption optics, using an An60-Ti rotor with cells containing quartz windows, as described previously (Domagala et al., 2000). Centrifugation experiments were conducted at 20°C using a sample volume of 100 µl. Equilibrium sedimentation distributions obtained at 12,000 and 20,000 rpm, were monitored at 280 or 290 nm and analysed using the program SEDEQ1B (Minton 1994). The partial specific volume of EGF was taken as 0.71 ml/g (Domagala, 2000).

Chemical Cross Linking - Chemically cross-linked sEGFR501 dimers were generated by the incubation of sEGFR501 (5 µM) with mEGF (20 µM) in 20 mM HEPES pH7.4 containing 150 mM NaCl for 1h at room temperature followed by the addition of bis(sulfosuccinimidyl)suberate (BS3, Pierce, Rockford, IL, USA) to a final concentration of 0.5 mM and incubation for a further 30 min. The reaction was terminated by the addition of Tris-HCl buffer (pH 7.5) to a final concentration of 10 mM. Monomer-dimer separation was achieved on Novex non-reducing SDS-PAGE gels (10%). Proteins were transferred onto poly(vinyl difluoride) (PVDF) membranes (Bio-Rad, Hercules, CA, USA) and identified by incubation with anti-EGFR Mab528 (Gill et al., 1984) (0.5 µg/ml) followed by horseradish-peroxidase labelled goat anti-mouse IgG (Bio-Rad) and ECL detection (Amersham Pharmacia Biotech).

Cell Proliferation Assays - BaF/3ERX cells, a cell line derived from BaF/3 cells transfected with human EGFR (a gift from Dr F.Walker, LICR, Melbourne) were washed three times to remove residual IL-3 and resuspended in RPMI 1640 + 10% FCS. Cells were seeded into 96 well plates using the Biomek 2000 (Beckman) at $2x10^4$ cells per 200 µl and incubated for

4 h at 37°C in 10% CO2. Appropriate concentrations of Mab528, sEGFR501 or sEGFR621 were added to the first titration point and titrated in two-fold dilutions across the 96 well plate in duplicate with or without a constant amount of mEGF (207 pM). 3H-Thymidine (0.5 μCi/well) was added and the plates were incubated for 20 h at 37°C in 5% CO_2. The cells were then lysed in 0.5 M NaOH at room temperature for 30 min before harvesting onto nitrocellulose filter mats using an automatic harvester (Tomtec, Connecticut, USA). The mats were dried in a microwave, placed in a plastic counting bag and scintillant (10 ml) was added. 3H-Thymidine incorporation was determined using an automated beta counter (1205 Betaplate, Wallac, Finland)

RESULTS

Production and Purification of Truncated EGFR Ectodomains – Preliminary analysis of conditioned media from cells transiently expressing sEGFR476, sEGFR501 and sEGFR513 showed that only the latter two produced truncated receptors which gave detectable binding to EGF on the BIAcore biosensor (data not shown). Stably transfected Lec8 cells expressing sEGFR501, were generated and used to produce truncated receptor protein at a yield of ~1.8 mg/L of fermentation medium.

Protein purified from a Mab9E10 anti-c-myc peptide affinity column, by peptide elution, showed a single symmetrical peak on size exclusion chromatography with an apparent molecular mass of ~80 kDa (Figure 3). The protein migrated as a single band of ~70 kDa on reduced SDS-PAGE (Figure 3 inset). N-terminal amino acid sequence analysis of the purified protein gave the single sequence, LEEKKVXQGT, corresponding to the N-terminal sequence of hEGFR receptor (Ullrich et al., 1984). The X at cycle 8 is due to the presence of a disulphide-bonded cysteine residue at that position. Additional protein, with properties similar to the peptide-eluted receptor was recovered from the Mab column by elution with x mM sodium citrate buffer, pH3, giving a further yield of ~0.7 mg/L.

The molecular mass (70 kDa) of the protein band on SDS-PAGE suggests significant glycosylation, since the calculated mass of human sEGFR501 apo-protein is ~57.5 kDa. There are eight potential N-linked glycosylation sites in sEGFR501 (Ullrich et al., 1984). Incubation of sEGFR501 with peptide-N-

glycosidase (PNG'ase) at 37° C resulted in the generation of a major band migrating on SDS-PAGE with an apparent molecular mass of ??? (data not shown), in good agreement with that of the apo-protein and consistent with N-linked glycosylation of the truncated receptor ectodomain.

Analytical Ultracentrifugation - Analytical ultracentrifugation was used to further analyze the purity and molecular weight distributions of the interacting species in sEGFR501: mEGF reaction mixtures (Figure 4A). The data for 20 mM EGF alone indicate a single solute of molecular weight 5,980 Da, in good agreement with the value calculated from the amino acid composition (6,040 Da). The molecular weight and partial specific volume determined for 10 mM sEGFR501 alone (65,600 Da and 0.71 ml/g) was calculated from the sedimentation equilibrium distribution and is based on the known amino acid composition and a calculated value of 12% (w/w) for the carbohydrate composition.

Sedimentation equilibrium data for a mixture of EGF (20 μM) and sEGFR501 (10 μM) was analyzed assuming two species (Figure 4A). The molecular weight of the first species was fixed at the value obtained for free EGF (6,000 Da) with the molecular weight and weight fraction of the second species used as fitting parameters. Under these conditions the molecular weight of the second species provides a good approximation to the weight-average molecular weight of sEGFR501 and its complexes. The best-fit value showed a complex of weight-average MW 106,400 Da, higher than predicted for a 1:1 complex (71,600 Da) and more consistent with the formation of a significant proportion of dimeric 2:2 EGF/sEGFR501 complex.

High speed meniscus depletion experiments were performed to determine the molar ratio required for saturation of sEGFR501 with EGF (Figure 4B). A solution of sEGFR501 (5 μM) was titrated with EGF to determine the molar ratio at which free EGF is detectable at the meniscus. The results show that this occurs above 5 μM EGF, implying an equimolar ratio is required for saturation of the EGF binding site(s) on sEGFR501. Taken together with the observed weight average molecular weight of the EGF/sEGFR501 complex obtained from the equilibrium analysis (Figure 4a), these data confirm that the stoichiometry of the dimeric species is 2:2 not 2:1.

The same approach was used for the analysis of data obtained for sEGFR501 (5 μM) in the presence of a range of EGF concentrations (Figure 4C). The weight average molecular weight obtained for the "second" species increases as the ratio of EGF/sEGFR501 is increased with the weight average molecular weight plateauing at approximately 110,000 at ratios above 2:1. The data in Figure 4A could also be fitted assuming a mixture of 1:1 and 2:2 complexes with weight fractions of the monomeric and dimeric sEGFR501 complexes of 57% and 31% respectively.

Chemical Cross-Linking – Further evidence of the ability of sEGFR501 to form a dimeric complex in the presence of ligand was obtained in chemical cross-linking experiments (Figure 5). Analysis by non-reducing SDS-PAGE was necessary since the antibody used to detect sEGFR501 (Mab528) is conformationally dependent. In the presence of mEGF (20 μM), a single high molecular weight species (apparent Mr 180,000 Da) was formed after chemical cross-linking which was not detectable when the cross-linking was attempted in the absence of ligand. Size exclusion chromatographic analysis of the reaction mixture, using a TSK G2000SW column developed with a mobile phase of PBS at a flow rate of 0.25ml/min, showed a peak of apparent Mr 158,000 which corresponded to a dimer (data not shown).

BIAcore Analysis Using Surface Plasmon Resonance Detection - The BIAcore biosensor was used to determine both the rate and equilibrium binding constants for the interaction between hEGF or hTGF-α and sEGFR501. sEGFR621 was used as a positive control for the surface reactivity, since this interaction has been studied in some detail previously (Domagala et al., 2000; Zhou et al., 1993). RP-HPLC-purified hEGF or hTGF-α were immobilised onto the sensor surface on adjacent sensor channels using amine coupling (typically 100 - 200 RU immobilised equivalent to 0.1–0.2 ng/mm^2 (Nice et al., 1994)). For kinetic and therodynamic analyses, binding curves were obtained by passing varying concentrations of sEGFR501 (10-100 nM) sequentially over the sensor surfaces.

Representative sensorgrams are shown in Figure 6. Visual inspection revealed that the curves approached equilibrium over the ranges tested. Additionally, the hTGF-α sensorgram appeared to show more rapid, and virtually complete dissociation. Therodynamic analysis of the equilibrium

binding data in Scatchard format (Figure 7) indicated KD values of 30 and 47 nM (correlation coefficient R=0.993 and 0.999 respectively) for the interactions between sEGFR501 and immobilised hEGF or hTGF-α and 412 and 961 nM (R=0.997 and 0.999 respectively) for the corresponding interactions with sEGFR621. The value observed for the interaction between sEGFR621 and hEGF is in good agreement with values reported previously (Greenfield et al., 1989; Grimaux et al., 1989; Lax et al., 1991; Zhou et al., 1993; Brown et al., 1994; Lemmon et al., 1997, Domagala et al., 2000). The values obtained by Scatchard transformation were also confirmed by direct non-linear least squares analysis of the binding data (data not shown) using an equation defining steady state equilibrium (KA*Conc*Rmax /(KA*Conc*n-1); BIAevaluation 3.1). Using this analysis, KD values of 32 and 46 nM were calculated for the interaction between sEGFR501 and immobilised hEGF and hTGF-α respectively and 570 and 959 nM for the interaction between full length ectodomain (sEGFR621) and immobilised hEGF and hTGF-α .

The individual rate constants were determined from those parts of the curves where first order kinetics appeared to be operative (Nice and Catimel, 1999), and the corresponding dissociation constants calculated (Table 1). Again, there was good agreement between the KD values calculated in this manner, and those obtained from the equilibrium binding data. The rate constants obtained for the interaction between sEGFR621 and immobilised hEGF were also similar to those obtained previously (apparent k_a of 2.0 $x10^5$ M^{-1} s^{-1} and kd of 0.05 s^{-1}, Domagala et al., 2000). It is interesting to note that the binding curves obtained with both sEGFR501 and sEGFR621 for hTGF-α appeared to be better fitted to a 1:1 model than the corresponding data for the hEGF surface (as suggested by the virtually complete dissociation).

Table 1 Comparative kinetic data for ligand binding by truncated and full-length EGFR ectodomains.

Interaction	k_a ($M^{-1}s^{-1}$) x 10^{-5}	k_d (s^{-1})	K_D (nM)
sEGFR501/EGF	10 – 17	0.02	13 – 21
sEGFR501/TGF-α	9.3 – 10.5	0.04	35 – 40
sEGFR621/EGF	2.9 – 4.8	0.08	180 – 300
sEGFR621/TGF-α	0.7 – 1.0	0.08	840 – 1320

Antagonist Activity - The observation that sEGFR501 bound to EGF with higher affinity than sEGFR621 prompted us to test whether sEGFR501 would act as a competitive inhibitor for the mitogenic stimulation of EGFR in a cell-based assay using the BaF/3ERX cell line. This cell line responds to mEGF with an EC_{50} of approximately 30 pM (Figure 8A). The competition assay (Figure 8B) used a constant concentration of mEGF (207 pM), which causes maximal stimulation (Figure 8A), and varying levels (0.00045–0.5 μM) of sEGFR501, sEGFR621 or the neutralising monoclonal antibody Mab528 (Gill et al., 1984). This antibody has been shown to prevent the growth of A431 cell xenografts, bearing high numbers of EGF receptors, in nude mice (Mendelsohn, 1988). The sEGFR501 (IC_{50}=0.02 μM) was almost 10 fold more potent than the full-length ectodomain (IC_{50}=0.15 μM) and approximately 3-fold more potent than the neutralising monoclonal antibody (IC_{50}=0.06 μM).

Biosensor Analysis of sEGFR501 mutants. – Biosensor analysis was also used to analyse the binding of the transiently expressed sEGFR501 mutants to both immobilised hEGF and hTGF-α surfaces. The presence of the mutant proteins in culture supernatants from transfected cell lines was demonstrated by both immunoblotting with the anti-EGFR monoclonal antibody, Mab 528, and biosensor analysis using Mab 528 immobilised on the surface (data not shown). Culture supernatants from all cell lines showed demonstrable binding to the Mab surface (441>472=wt>367).

When passed over the hEGF sensor surface, the Glu367Lys mutant (not shown) and the Glu472Lys mutant showed similar binding characteristics to sEGFR501 (Figure 9A). The Gly441Lys mutant showed much reduced binding, even though the Mab528 surface had indicated that the Gly441Lys mutant was present at higher concentrations than sEGFR501. Interestingly, when the same samples were passed over the parallel hTGF-α sensor surface the Gly441Lys mutant now showed the highest binding, whilst the binding of the Glu367Lys mutant (not shown), the Glu472Lys mutant and wild type sEGFR501 was lower (Figure 9B). The reduced binding of the transiently expressed sEGFR501 preparation to the TGF-α surface in these experiments is in good agreement with the data obtained with the purified protein (Figure 7 and Table 1).

DISCUSSION

The characteristics of a truncated version of the EGFR ectodomain that binds hEGF and hTGF-α with high affinity (Figure 7 and Table 1) is described herein. The K_D values of 13-21 nM for EGF are similar to those, (15–30 nM), seen with chemically cross-linked dimers of full-length EGFR ectodomain (Hurwitz et al., 1991; Zhou et al., 1993) and are 10 to 25-fold higher than the values (150–400 nM) generally reported for soluble, full-length EGFR ectodomain from either A431 tumour cells (Grimaux et al., 1989; Brown et al., 1994), transfected Sf9 insect cells (Greenfield et al., 1989; Hurwitz et al., 1991; Brown et al., 1994) or CHO cells (Lax et al., 1991; Zhou et al., 1993; Lemmon et al., 1997; Domagala et al., 2000). Recently it was shown that preparations of full-length EGFR ectodomain contained two populations of molecules, one of which showed high affinity (K_D 1-20 nM) for EGF and represented 10-15% of the total preparation (Domagala et al., 2000). The nature of the higher affinity form was not established.

The truncated ectodomain described here, sEGFR501, comprises the L1/CR1/L2 domains plus the first module of the second Cys-rich region CR2. Its design was based on the following information. Firstly, the shorter construct, sEGFR476, comprising the L1/CR1/L2 domains only, failed to bind ligand suggesting that additional regions of the receptor were required. Secondly, the structure of the L1/CR/L2 fragment of IGF-1R showed that a conserved tryptophan residue, Trp176, in the first module of the Cys-rich

region, is inserted between the last two rungs of the L1 domain. (Garrett et al., 1998). The importance of this structurally was suggested by the observation that a tryptophan residue, Trp492, is present in the corresponding position in the first module of the second Cys-rich region of EGFR (Garrett et al., 1998). The residues forming the hydrophobic pocket are conserved in the L2 domain of the EGFR but not in the L2 domains of IGF-1R and IR, which lack a second Cys-rich region (Garrett et al., 1998). sEGFR501 was constructed to retain the first module of the second Cys-rich region and the conserved Trp492, predicted to stabilise the structure of the L2 domain of the EGFR (Garrett et al., 1998; Jorissen et al., 2000). Thirdly, the normal ligand binding and signalling properties of an 83 amino acid (residues 521-603) deletion mutant of EGFR from a human glioma (Humphrey et al., 1991) indicated that much of the CR2 domain (domain IV) could be removed without detriment. This deletion extends from the middle of the third module to the middle of the seventh (last) module of the second Cys-rich region of hEGFR and disrupts three disulphide bonds (Ward et al., 1995; Garrett et al., 1998; Jorissen et al., 2000). Surprisingly, deletion of 72 residues from the same region, (amino acids 518-589, disrupts only two disulphide bonds), essentially abolished EGF binding and EGF-induced autophosphorylation (Saxon and Lee, 1999). Fourthly, the 40 kDa, proteolytically-derived, EGFR fragment (residues 302-503), which is capable of binding EGF, also includes the first module of CR2 (Kohda et al., 1993). Finally, the naturally occurring EGFR with a mutation Arg497Lys in the first module of CR2, binds EGF normally but shows only low affinity binding of TGF-α (Moriai et al., 1994). These observations suggest the importance of the interactions between this module and the L2 domain.

The high affinity binding of sEGFR501 suggests that the loss of EGF binding seen in the EGFR with multiple mutations (Glu521Ala, Arg523Ala, Glu524Ala, Phe525Ala, Glu527Ala), in the third module of CR2 (domain IV) or after deletion of residues 518-589 (Saxon and Lee, 1999), is due to indirect effects on receptor orientation rather than a direct contribution to the ligand-binding pocket. Molecular modelling (Jorissen et al., 2000) indicates that the first module of CR2 (residues 480-501, Figure 1), is unlikely to make direct contact with ligand, since it is positioned away from the putative binding pocket and away from the face of the L2 domain implicated in ligand binding by chemical cross-linking studies (Summerfield et al., 1996).

sEGFR501, which lacks most of CR2, is capable of high affinity binding of both hEGF and hTGF-α (Figs 6 and 7), and exhibits ligand-induced receptor dimerisation (Figs 4 and 5) at much lower receptor concentrations than reported previously for sEGFR621 (Cadena and Gill, 1993; Tanner and Kyte, 1999; Lemmon et al., 1997). The receptor regions responsible for dimerisation have not been identified but, are unlikely to include CR2 (domain IV) based on our findings and the data reported for the EGFR domain IV deletion mutant (Humphrey et al., 1991). The ultracentrifugation analyses at different molar ratios of sEGFR501 and EGF showed that the binding sites on sEGFR501 were saturated and the extent of dimerisation began to plateau at molar ratios greater than of 1:1(Figure 4C), even at the relatively low concentration of sEGFR501 of 5μM (320 μg /ml). This compares favourably with small angle X-ray scattering data that showed that sEGFR621 dimerisation, induced by EGF or TGF-α binding, reached a maximum when the ratio of EGF/sEGFR was 1:1 (Lemmon et al., 1997). These studies were carried out at relatively high concentrations (60-110 μM; 5-9 mg/ml) of receptor ectodomain (Lemmon et al., 1997). Other studies of EGF-induced sEGFR621 dimerisation have indicated the importance of receptor concentration, with no dimers being observed at the low concentrations (<100 nM) frequently used for binding studies (Greenfield et al., 1989) or, in some cases, at considerably higher concentrations of 5-30 μM (Cadena and Gill., 1993; Tanner and Kyte, 1999). Previous analytical ultracentrifugation and chemical cross-linking studies showed that 30-40% of sEGFR621 existed as dimers when the ratio of EGF/sEGFR621 was 2:1 and the starting concentration of sEGFR621 was ~ 17 μM (1.33 mg/ml) (Brown et al., 1994), three-fold higher than the concentrations used in the studies with sEGFR501 reported here.

The enhanced capacity of sEGFR501 (compared to sEGFR621), to form dimers on binding ligand, suggests that part of the second Cys-rich domain (domain IV) sterically interferes with dimerization in the full-length ectodomain. It was rercently shown that detergent solubilised EGFR ectodomain containing additionally only the transmembrane region, but no cytoplasmic domain, readily formed dimers on binding EGF, at nM receptor concentrations (Tanner and Kyte, 1999), suggesting that membrane tethering alters the relative position of CR2 in the transmembrane-anchored ectodomain construct. This is reminiscent of the effects of transmembrane

anchoring, or C-terminal tethering, on the generation of curvilinear Scatchard plots and high affinity binding by the insulin receptor ectodomain (Whittaker et al., 1994; Bass et al., 1996). Normal insulin receptor ectodomain displays only linear Scatchards and low affinity binding. The maximum extent of dimerization seen with the solubilised, membrane anchored EGFR ectodomain in the presence of excess EGF, was 45-50% (Tanner and Kyte, 1999).

In this study sEGFR501-based mutants were used to investigate the molecular basis of the differential binding of hEGF and hTGF-α by chicken EGFR (Lax et al., 1989). Chicken EGFR binds hTGF-α as well as, or better than, hEGFR but binds hEGF and mEGF with approximately 100-fold lower affinity compared to the human receptor (Lax et al., 1988). This is in contrast to the murine and human EGFR which bind hEGF, mEGF and hTGF-α with high affinity despite the considerable differences in the sequences of the ligands (16-39 differences) and the receptors (72 differences) (Ullrich et al., 1984; Avivi et al., 1991).

The region on the ligand responsible for poor binding to the chicken receptor was mapped to the C-terminal residues 43-53, after examination of the binding properties of ten hEGF/ hTGF-α chimeras (Kramer et al., 1994). Subsequent analysis of this C-tail region, using a series of truncated ligands or multiple site/single site mutations (where the residues in hEGF were replaced by those found in hTGF-α), showed that Arg45 was critically important in preventing binding of hEGF to the chicken EGFR (van de Poll et al., 1995). The single mutation Arg45Ala was sufficient to confer high affinity binding of hEGF to the chicken EGFR. The low binding affinity of hEGF was suggested to result from electrostatic repulsion by positively-charged residues close to, or within, the ligand binding domain of the chicken receptor, but absent from the human or mouse receptors (van de Poll et al., 1995).

The region of the chicken receptor responsible for discrimination between mEGF and hTGF-α was identified as residues Lys301-Asp484 (mature receptor numbering) from an analysis of a series of chimeras of the human and chicken receptors (Lax et al., 1989). This region contains the L2 domain plus some small flanking sequences. It starts near the end of the seventh module of CR1 and ends at the beginning of the first module of CR2 (Ward et al., 1995; Garrett et al., 1998; Jorissen et al., 2000). There are 49

sequence differences between the chicken and human receptors in this region, six of which, (equivalent to residues 340, 367, 420, 441, 472 and 479 in hEGFR), are basic residues not present in the human or mouse receptor.

Molecular modelling (Figure 1), based on the 3D structure of the IGF-1R (Garrett et al., 1998; Jorissen et al., 2000), indicated that residue 340 is positioned at the back of the L2 domain and is unlikely to contribute to binding, while residue 420 is part of a glycosylation site in hEGFR and likely to be masked by carbohydrate. Residue 479 was not mutated since it lies between the end of the L2 domain and the first module of CR2 and appeared unlikely to be part of the binding site (Jorissen et al., 2000). The three residues mutated to lysine in this study were Glu367, Gly441 and Glu472 (Figure 1). Residue Glu367 is close to the epitope (residues 351-364) for three competitive antibodies LA-22, LA-58 and LA-90 (Wu et al., 1989) and had been suggested as the most likely candidate for discrimination against hEGF by the chicken receptor (van der Poll et al., 1995).

The Glu367Lys mutation had no effect on binding of either ligand. Residues Gly441 and Glu472 are on the same face of the L2 domain as Lys465, the residue labelled by chemically cross-linking an Arg45Lys mutant of hEGF (Summerfield et al., 1996). While the Glu472Lys mutation had no effect on ligand binding characteristics, the Gly441Lys mutation showed considerably reduced affinity for hEGF without compromising its affinity for hTGF-α (Figure 9). These data demonstrate that the Lys442 in chicken EGFR, which corresponds to Gly441 in hEGFR, is the residue responsible for discriminating against hEGF and interacting with Arg45 (Kramer et al., 1994, van der Poll et al., 1995).

The truncated receptor, sEGFR501, is a valuable reagent to further investigate the parameters involved in ligand binding and ligand-induced receptor homo- and hetero-dimerisation. It may have therapeutic potential given its high affinity for ligand and its ability to competitively inhibit EGF-induced proliferation responses in a model cell system (Figure 8). This inhibition was greater than that achieved with an inhibiting monoclonal antibody. Therapeutic antibodies against ErbB2 have been shown to be effective against some cancers (Hoyle, 1998).

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the

invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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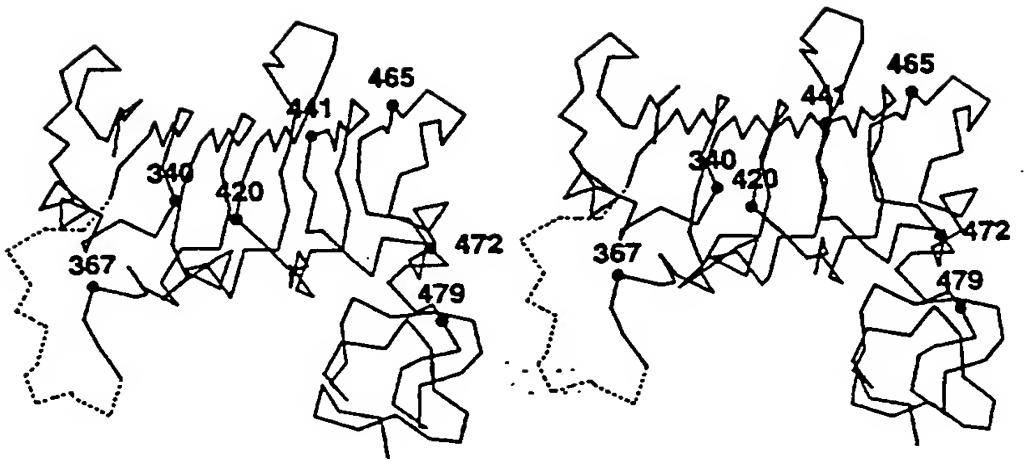


Figure 1

hEGFR 1-476 construct

```
                    476(  EK cleavage /          c-myc tail              /Stop)
                SerCysLysAspAspAspAspLysGluGlnLysLeuIleSerGluGluAspLeuAsn***
      5'-TAATCAGCTGCAAGGACGATGACGATAAGG- 3'
            Pvu II 5'.GACGATGACGATAAGGAACAAAAACTCATCTCAGAAGAGGATCTGAATTAG..3'
                   3'.CTGCTACTGCTATTCCTTGTTTTTGACTAGAGTCTTCTCCTAGACTTAATC...5'
                                                3' -CTTCTCCTAGACTTAATCTAGATATA- 5'
                                                                  Bgl II
```

hEGFR 1-501 construct

```
   496            501   (  EK cleavage /          c myc tail                /Stop)
   ProArgAspCysValSerAlaAspAspAspAspLysGluGlnLysLeuIleSerGluGluAspLeuAsn***<XbaI>    clamp
3'-GGGTCCCTGACGCAGAGCCGGCTGCTACTGCTATTCCTCGTCTTCGACTAGA-5' and
1744          1760                    3'-CTCGTCTTCGACTAGAGTCTTCTCCTAGACTTAATCAGATCTCCATGGGGC-5'
```

hEGFR 1-513 construct

```
   508             513(EK cleavage/        c myc tail                /Stop)
   GlyArgGluCysValAspAspAspAspLysGluGlnLysLeuIleSerGluGluAspLeuAsn***<XbaI>    clamp
3'-CCGTCCCTTACGCACCTGCTACTGCTATTCCTCGTCTTCGACTAGA-5' and
1780            1797          3'-CTCGTCTTCGACTAGAGTCTTCTCCTAGACTTAATCAGATCTCCATGGGGC-5'
```

Figure 2

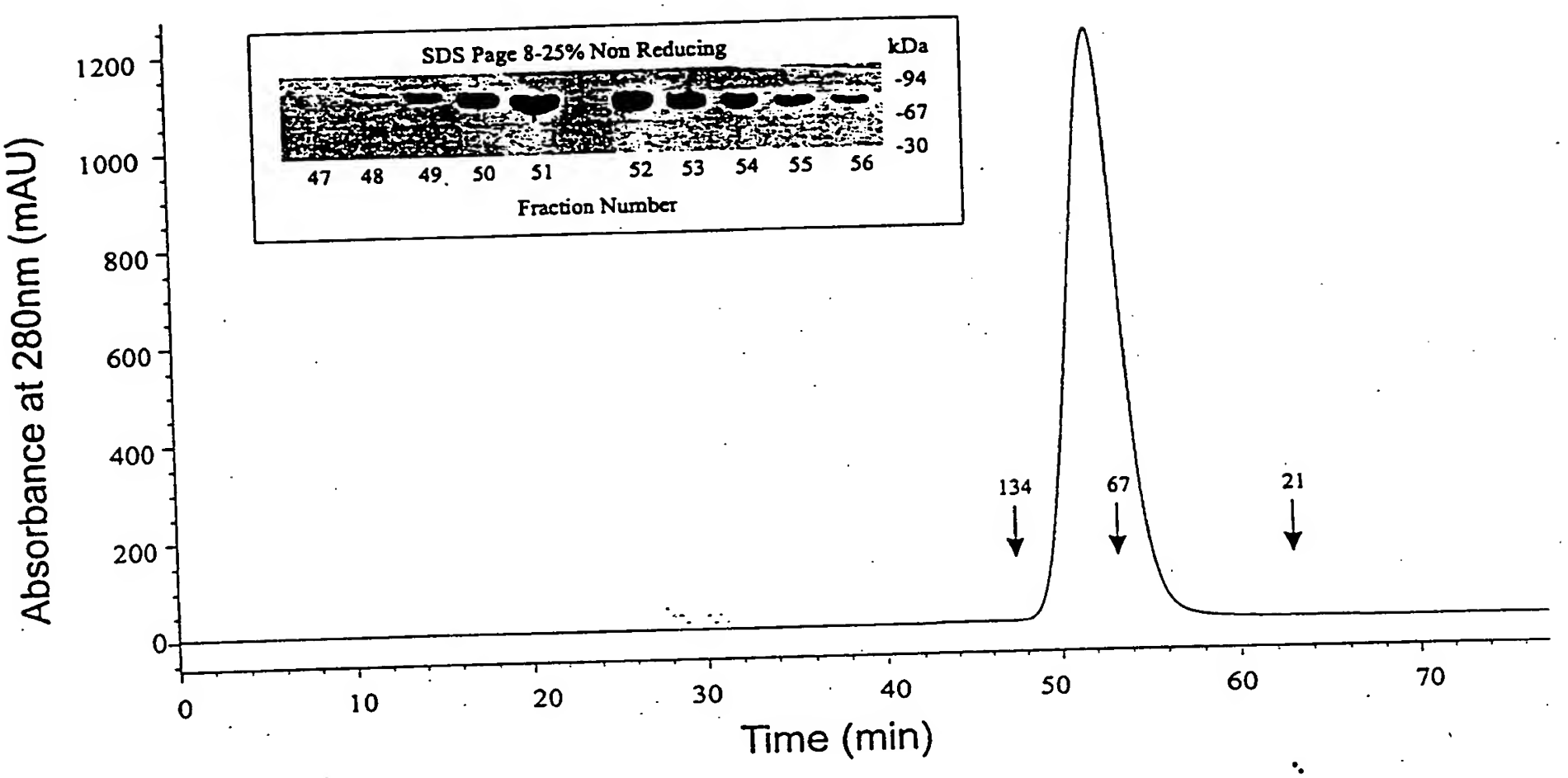
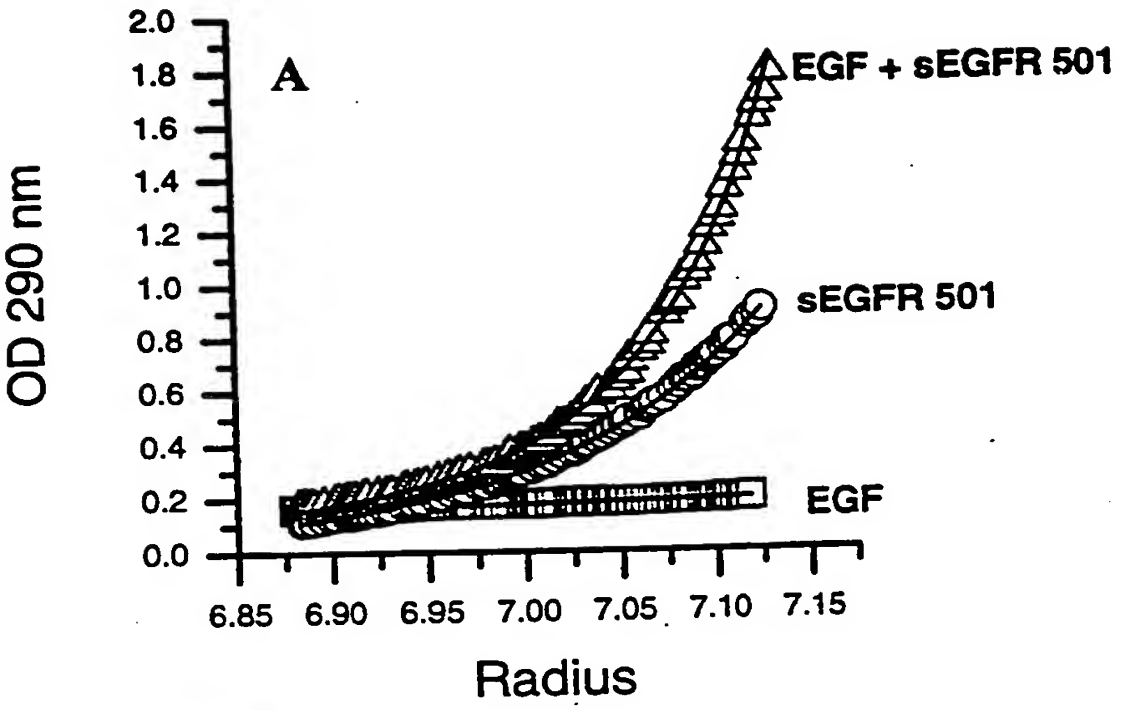
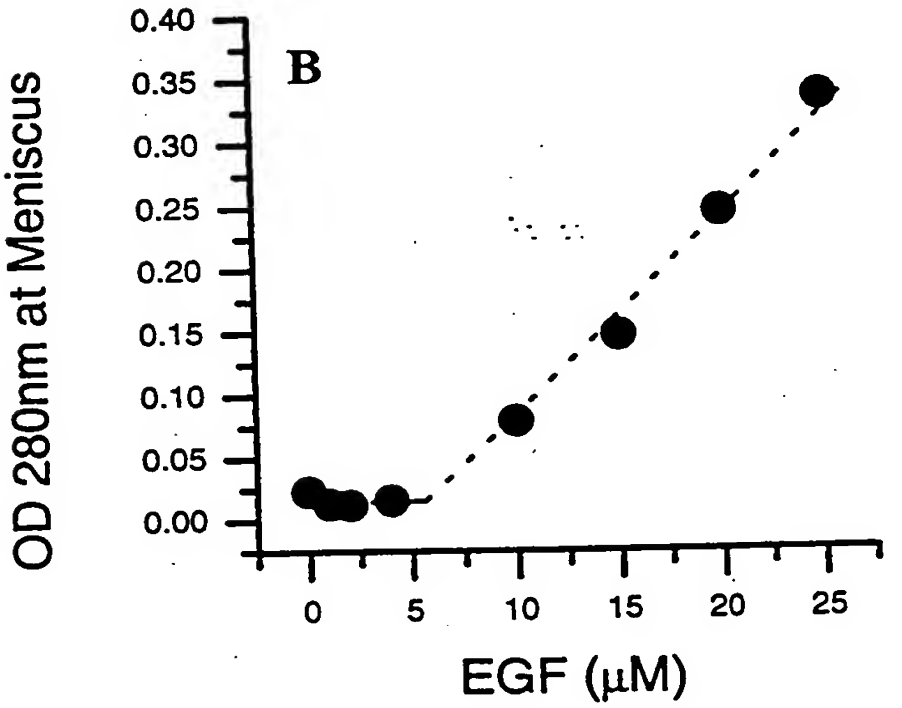

SDS Page 8-25% Non Reducing
kDa
-94
-67
-30
47 48 49 50 51 52 53 54 55 56
Fraction Number
Absorbance at 280nm (mAU)
1200
1000
800
600
400
200
0
0 10 20 30 40 50 60 70
Time (min)
134
67
21

Figure 3


A
EGF + sEGFR 501
sEGFR 501
EGF
OD 290 nm
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
6.85
6.90
6.95
7.00
7.05
7.10
7.15
Radius


B
OD 280nm at Meniscus
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
5
10
15
20
25
EGF (μM)

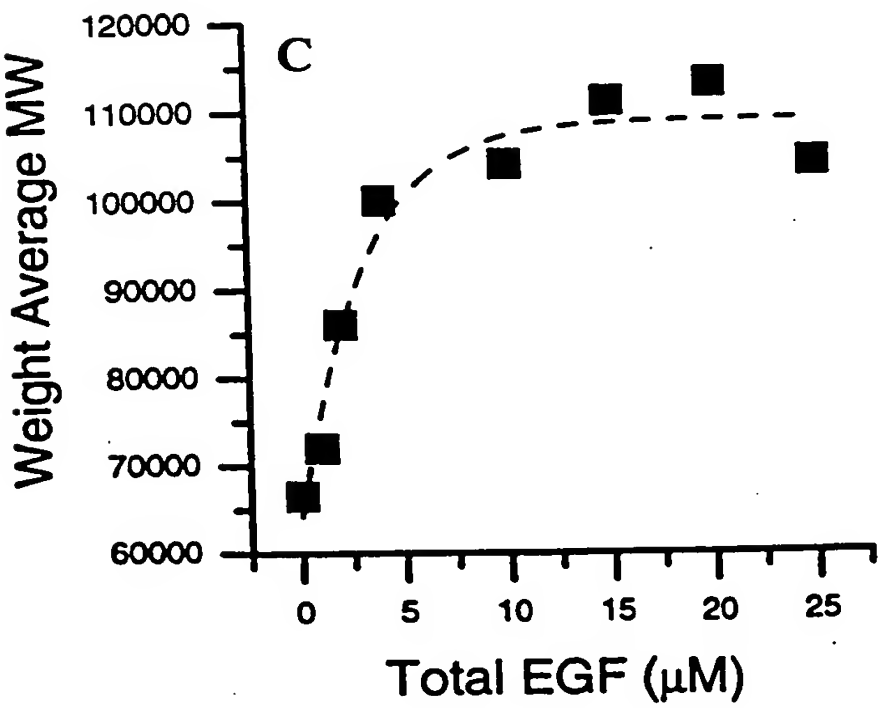

C
Weight Average MW
120000
110000
100000
90000
80000
70000
60000
0
5
10
15
20
25
Total EGF (μM)

Figure 4

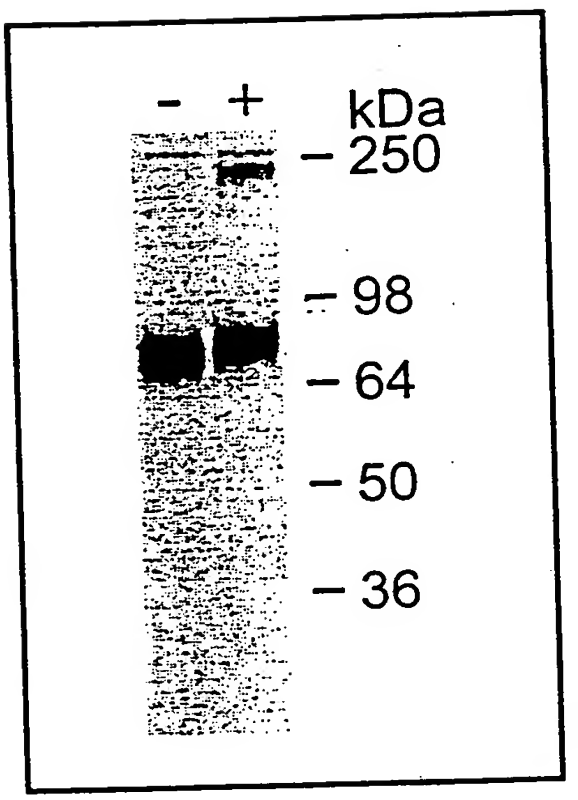


Figure 5

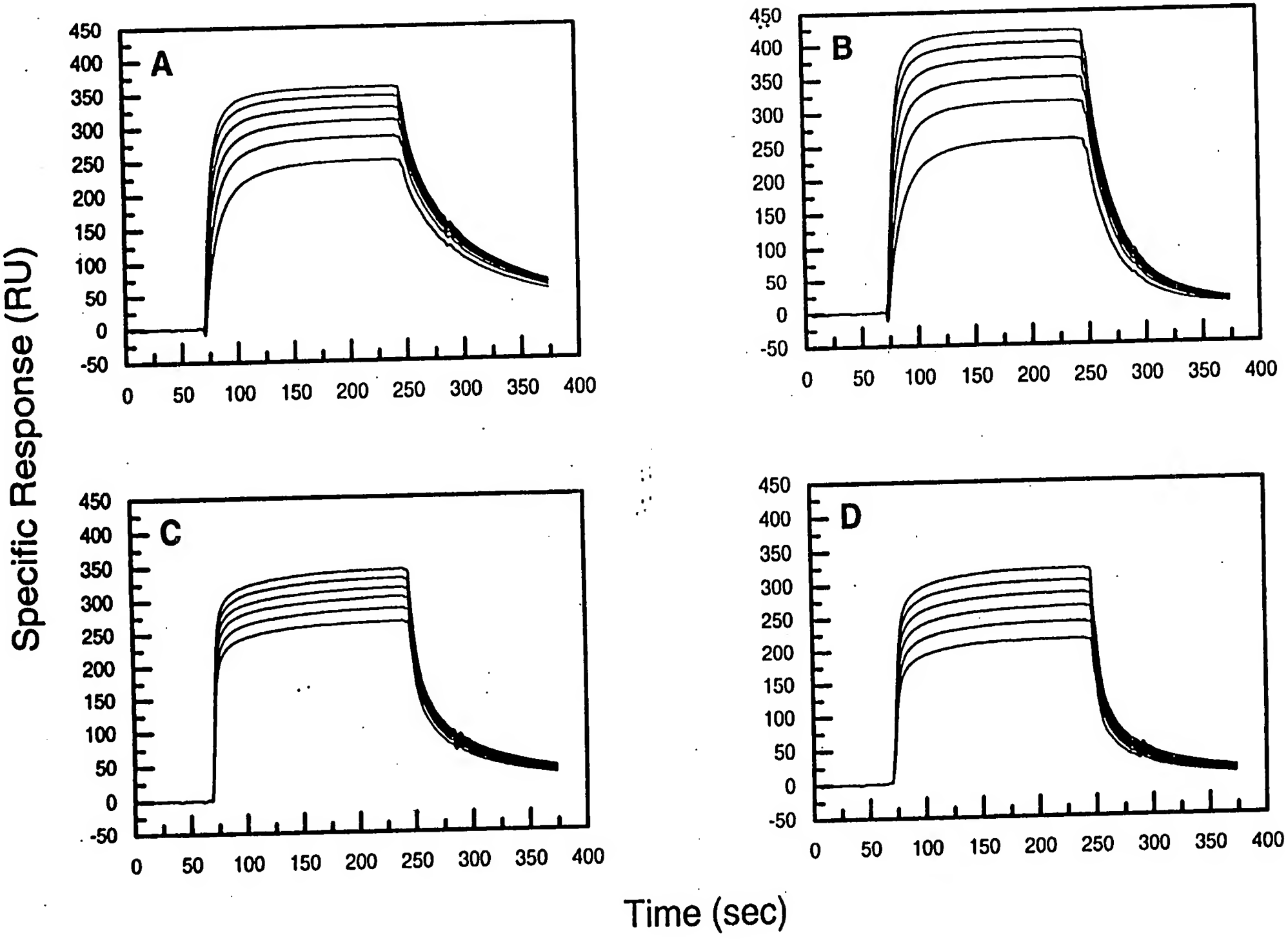

A
B
C
D
Specific Response (RU)
Time (sec)
450
400
350
300
250
200
150
100
50
0
-50
0
50
100
150
200
250
300
350
400

Figure 6

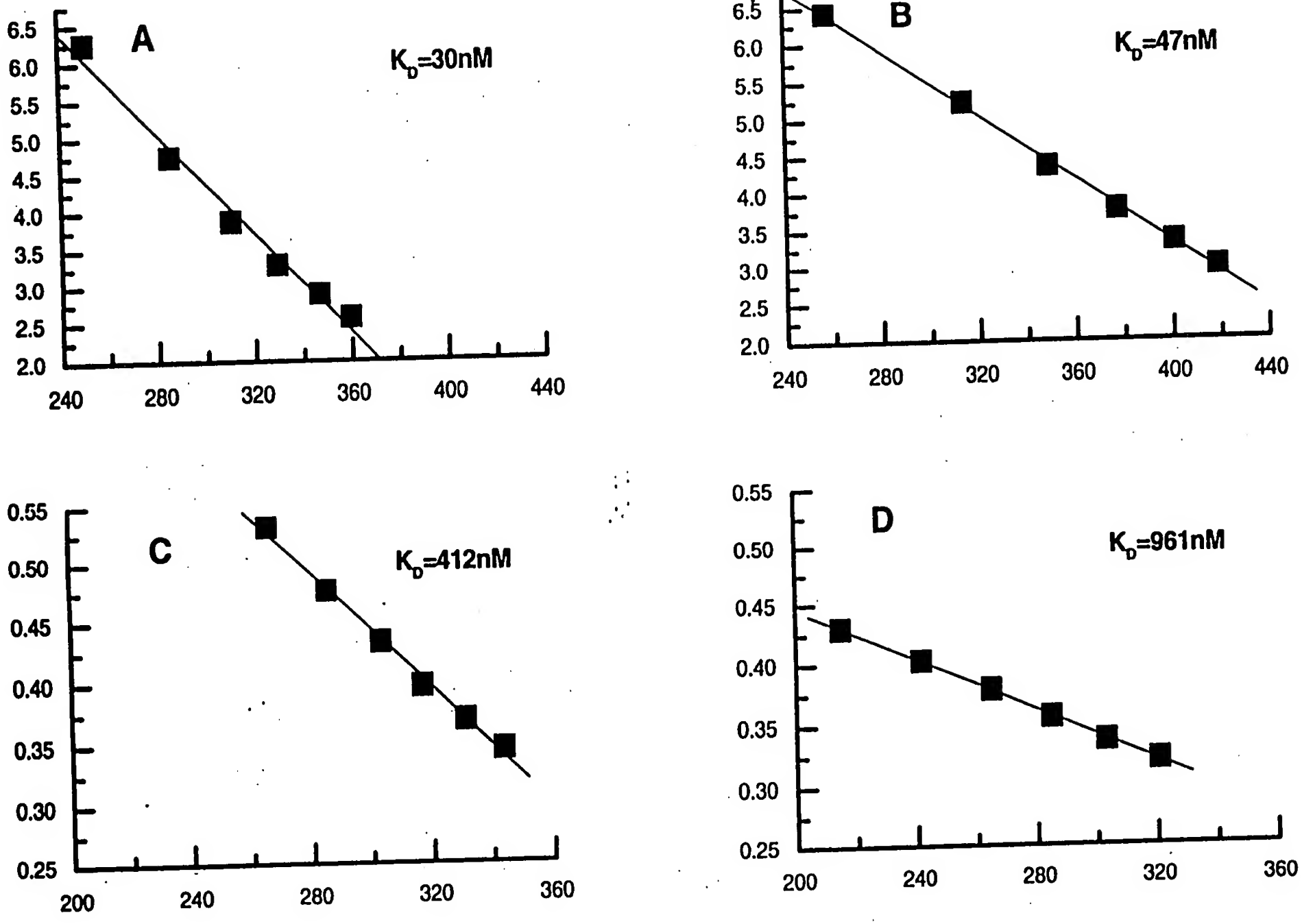
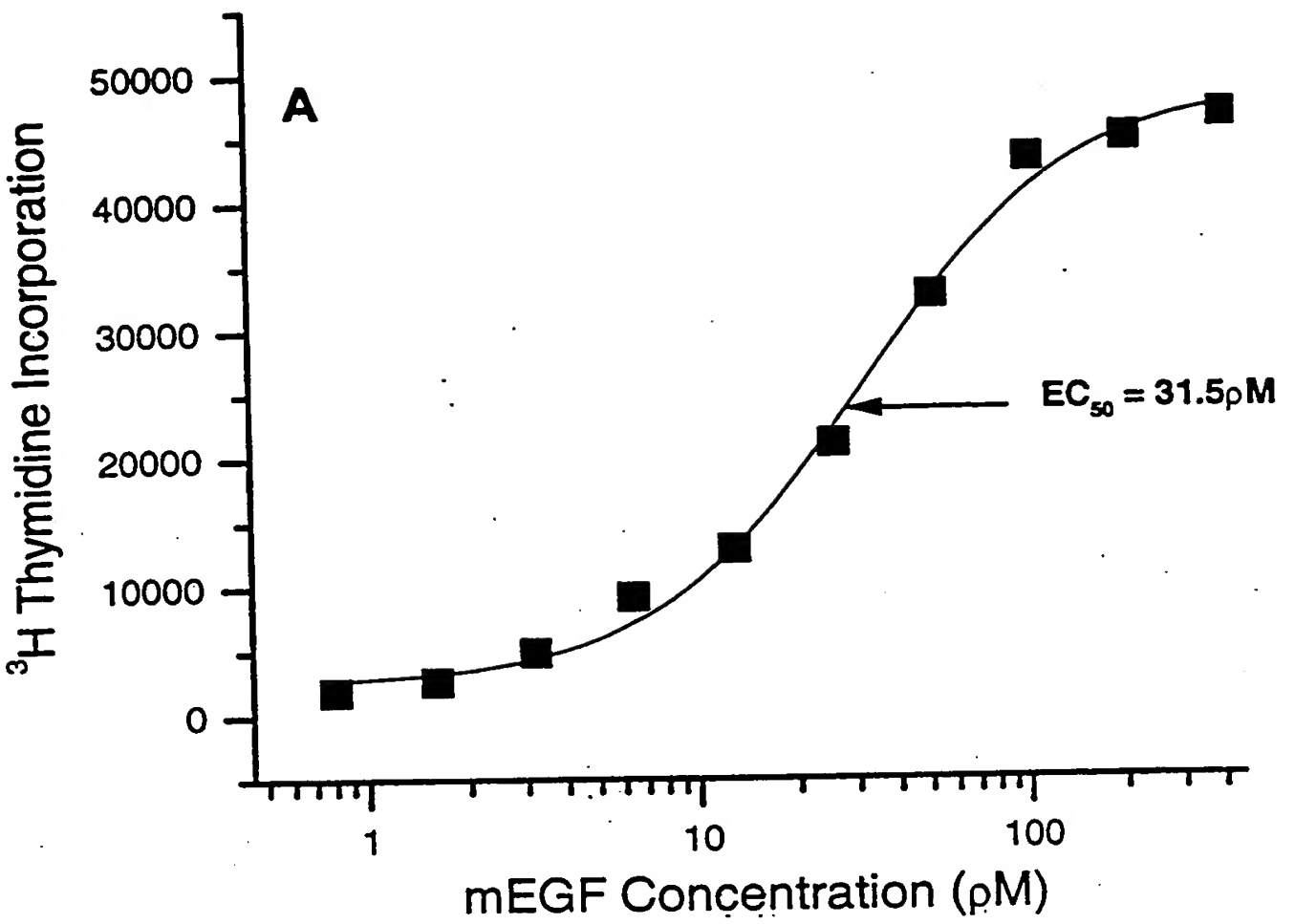


Figure 7


A
^{3}H Thymidine Incorporation
50000
40000
30000
20000
10000
0
1
10
100
mEGF Concentration (pM)
EC$_{50}$ = 31.5pM

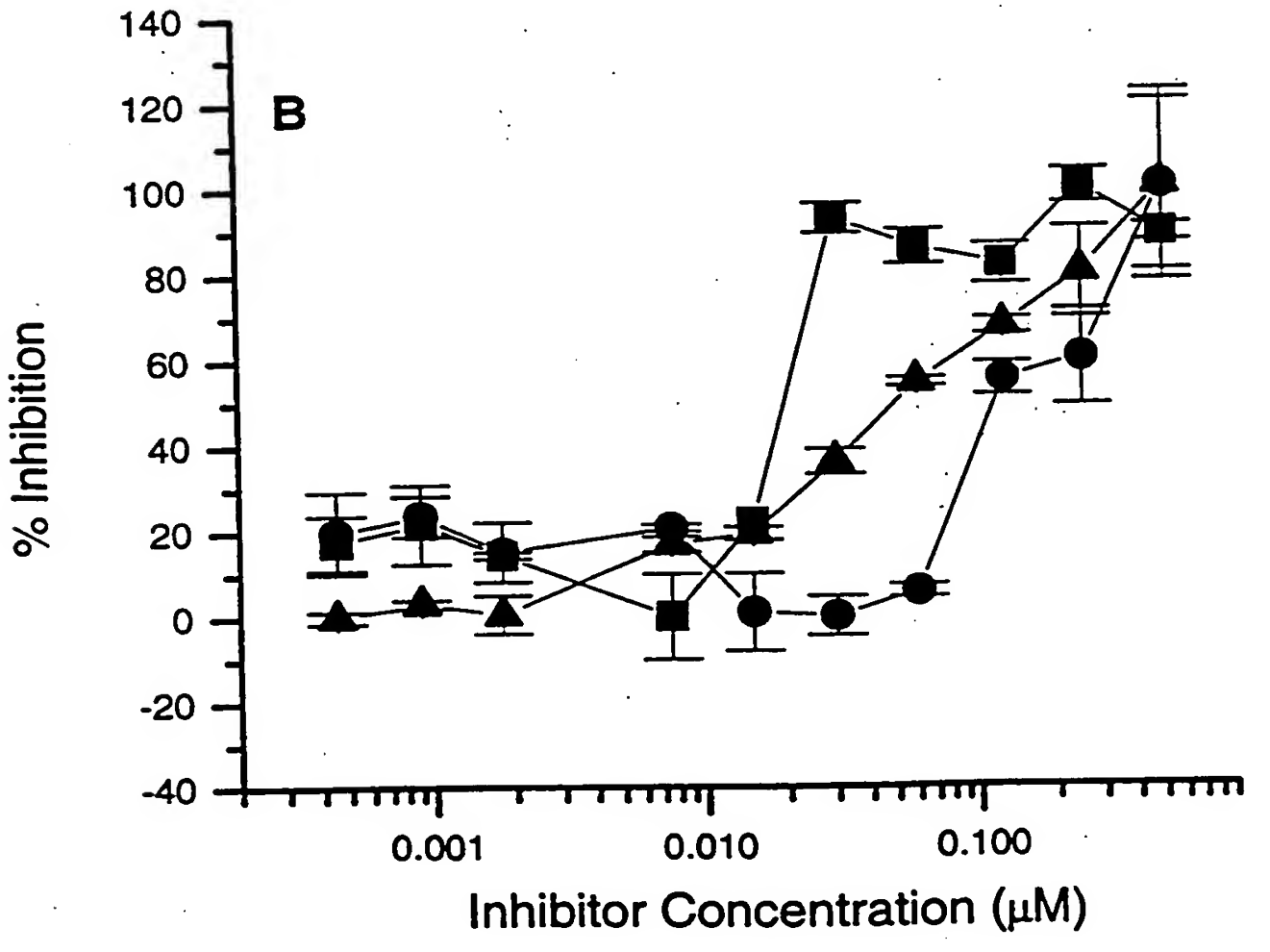

B
% Inhibition
140
120
100
80
60
40
20
0
-20
-40
0.001
0.010
0.100
Inhibitor Concentration (μM)

Figure 8

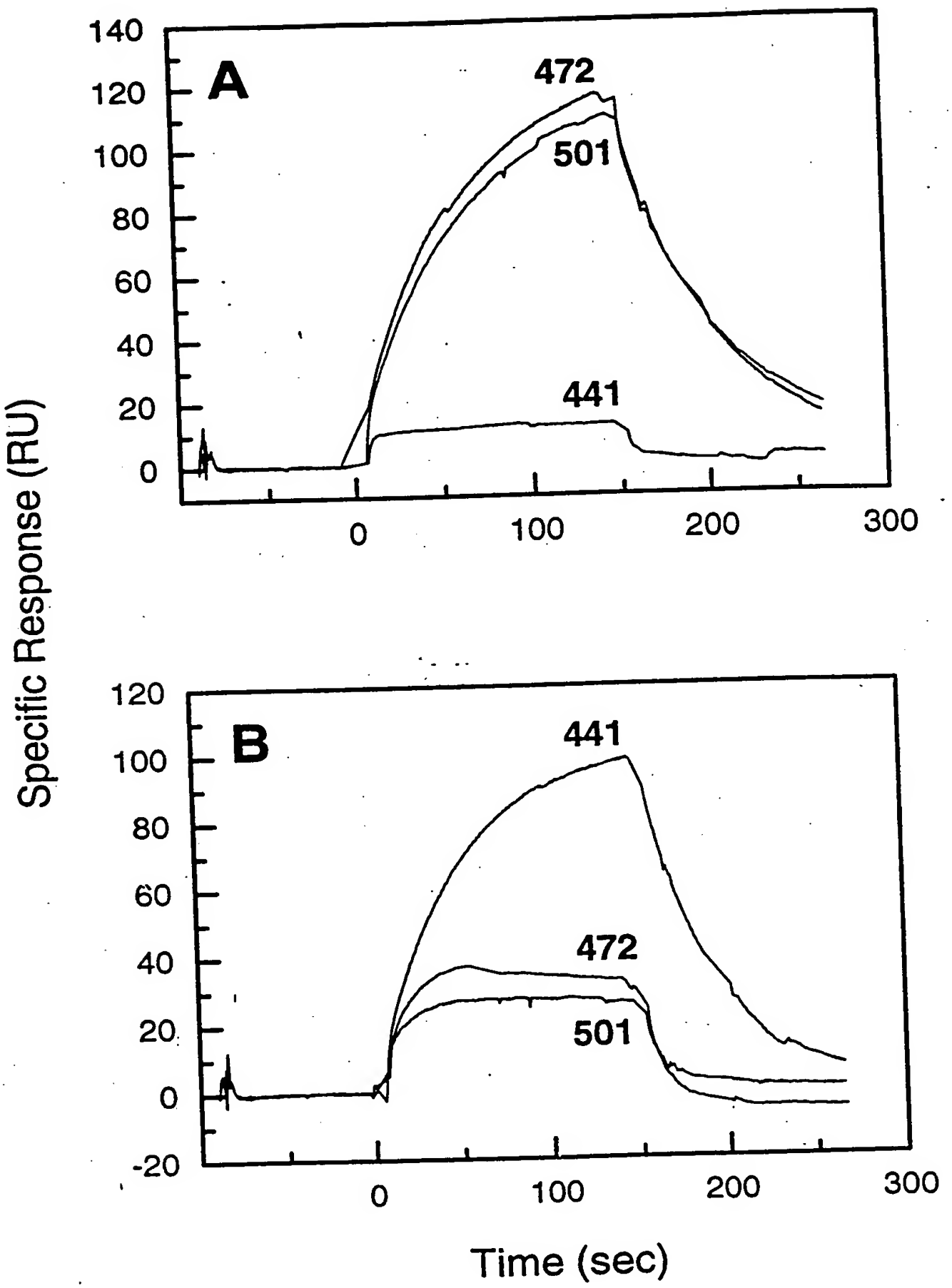

A
472
501
441
140
120
100
80
60
40
20
0
0
100
200
300
B
441
472
501
120
100
80
60
40
20
0
-20
0
100
200
300
Specific Response (RU)
Time (sec)

Figure 9